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Physiological responses and differential gene expression in *Prunus* rootstocks under iron deficiency conditions

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Physiological responses and differential gene expression in *Prunus* rootstocks under iron deficiency conditions

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Summary

Two *Prunus* rootstocks, the Myrobalan plum P 2175 and the interspecific peach-almond hybrid, Felinem, were studied to characterize their biochemical and molecular responses induced under iron deficient conditions. Plants of both genotypes were submitted to different treatments using a hydroponic system that permitted removal of Fe from the nutrient solution. Thus, Control plants were grown in 90μM Fe (III)-EDTA, Deficient plants were grown in an iron free solution, and plants submitted to an Inductor treatment were resupplied with 180 μM Fe (III)-EDTA during 1 and 2 days after a period of 4 or 15 days of growth on an iron-free solution. Felinem increased the activity of the iron chelate reductase (FC-R) in the Inductor treatment after 4 days of iron deprivation. In contrast, P 2175, did not show any response after at least 15 days without iron. The induction of the FC-R activity in this genotype was coincident in time with the medium acidification. These results suggested two different mechanisms of iron chlorosis tolerance in both Strategy I genotypes. Thus, Felinem would use the iron reduction as the main mechanism to capture the iron from the soil, meantime in P 2175 the mechanism of response would be slower and start with the acidification of the medium synchronized with the gradual loss of chlorophyll in leaves. In order to understand the control of these responses at the molecular level, the differential expression of *PFRO2*, *PIRT1* and *PAHA2* genes involved in the reductase activity, the iron transport in roots, and the proton release, respectively, were analyzed. The expression of these genes, estimated by quantitative real-time PCR, was different between genotypes and among treatments. Results were in agreement with the physiological responses observed, and are discussed through the paper.

Keywords: ferric chelate reductase, iron chlorosis**,** proton extrusion, qRT-PCR

Introduction

Iron chlorosis is a common nutritional disorder affecting a wide range of crops growing in calcareous soils where the iron is present predominantly in an insoluble form that can not be assimilated by the plant. The main symptom of iron chlorosis is the interveinal yellowing of younger leaves as a result of inhibition in the chlorophyll synthesis, which reduces the photosynthetic rate and subsequent growth of the plant (Marsh et al., 1963). Cultivated plants differ widely in their susceptibility to Fe deficiency in calcareous soils.

Among others, the iron chlorosis has a strong incidence in fruit crops of high economic importance since a significant part of the fruit tree industry in Europe and especially in the Mediterranean area is located on calcareous or alkaline soils. The occurrence of this nutritional disorder in crops like peach, pear, quince and kiwifruit affect fruit quality being responsible of enormous economic losses (Tagliavini and Rombola, 2001). The use of tolerant Fe-efficient rootstocks represents the best alternative to prevent iron chlorosis in these species (Tagliavini and Rombolà, 2001; Gogorcena et al., 2004; Jiménez et al., 2008). Nowadays, breeding programs for *Prunus* rootstocks have been directed to generate complex hybrids, with the maximum of desirable traits, to overcome soil and disease problems. For that reason, some interspecific hybrids were produced to generate high-performance rootstocks for Mediterranean environments by combination of their complementary traits (Dirlewanger et al., 2004; Felipe, 2009).

It is well known that efficient plants submitted to iron shortage conditions develop specific mechanisms in order to mitigate the lack of this essential nutrient. Thus, Strategy I plants (dicots and non-gramineous monocots) respond to iron deprivation with both morphological and physiological changes (Römheld, 1987). Morphological modifications include the development of lateral roots, formation of root hairs and differentiation of specialized transfer cells. These changes are oriented to increase the surface area between the plant and the soil and consequently, roots become more effective for Fe uptake (Gogorcena et al., 2001). Moreover, Strategy I plants have developed physiological changes such as the increase of the iron reductase capacity and the proton

extrusion in the rhizosphere region (for review, see Schmidt, 1999). Under iron starvation conditions, the modification in the expression of genes involved in iron uptake and transport also occurs (Mukherjee et al., 2006; Buckhout et al., 2009; Ding et al., 2009; Santi and Schmidt, 2009). Some of the genes coding for components of the Strategy I iron acquisition response, as ferric chelate reductase (FC-R) and the iron transporters, have been identified in species such as *Arabidopsis thaliana*, cucumber (*Cucumis sativus*), *Medicago truncatula*, pea (*Pisum sativum*), tomato (*Solanum lycopersicum*) and peanut (*Arachis hypogaea*) (Eide et al., 1996; Robinson et al., 1999; Eckhardt et al., 2001; Vert et al., 2002; Waters et al., 2002; Li et al., 2004; López-Millán et al., 2004; Waters et al., 2007; Ding et al., 2009). Genes coding for H^+-ATPases have been described in species as cucumber and Arabidopsis (Santi et al., 2005; Santi and Schmidt, 2009). The adaptative mechanisms developed in plants to overcome the iron deficiency are mainly regulated at the transcriptional level (Buckhout et al., 2009). However, the molecular mechanisms underlying the Fe stress-induced responses are still not well characterized in plants. It is obvious than the breeding approach, to overcome this abiotic stress, would be more efficient if genetic components, controlling iron acquisition, transport and storage, were better known.

Many studies have indicated that similarly to herbaceous dicots, some *Prunus* genotypes are able to improve iron acquisition through adaptation mechanisms. Thus, several species of the *Prunus* genus increases the FC-R activity in conditions of iron depletion and/or induce the proton extrusion to improve the efficiency in iron uptake (Romera et al., 1991; Cinelli and Viti, 1995; Gogorcena et al., 2004; Molassiotis et al., 2006; Jiménez et al., 2008).

For that reason, the main objectives of the present work are to characterize the physiological responses under iron starvation of two *Prunus* genotypes used as parents in breeding programs and to analyze the differential expression of genes under iron deficiency conditions in order to use them as a tool to accelerate the breeding process of iron chlorosis tolerant genotypes selection.

Material and Methods

Plant material and growth conditions

Two *Prunus* rootstocks, the Myrobalan plum (*Prunus cerasifera* Ehrh) clone P 2175 and the almond-peach hybrid (*P. amygdalus* Batsch x *P. persica* L. Bastch) clone GxN 22 (Felinem), were studied. These genotypes were selected for their good performance to the Mediterranean growing conditions (Felipe, 2009) and their use in an European breeding program as parents of a F_1 population (Dirlewanger et al., 2004). Plants from Felinem and P 2175 were propagated in vitro in Agromillora Catalana S.A. (Subirats, Barcelona, Spain) and in the Centro de Investigación Agroalimentaria de Aragón, CITA (Zaragoza, Spain), respectively.

Two week old propagated plants from both genotypes were grown in hydroponic culture as it was described by Jiménez et al. (2008). Plants were cultivated in a growth chamber under controlled environmental conditions with a 16/8 h photoperiod at 220 μmol m^{-2} s^{-1} at 22/20ºC with the relative humidity maintained at 70% to 75%.

Ten plants per genotype and treatment were submitted to different conditions to understand their response under lack of iron: Control (90 μM Fe (III)-EDTA), Deficient (plants were grown in an iron free solution), and Inductor (plants growing in the absence of iron during 4 or 15 days were resupplied with 180 μM Fe (III)-EDTA during 1 and 2 days, respectively). Each experiment was repeated twice for both genotypes. The chlorophyll concentration was estimated measuring the third and fourth expanded leaves for each treatment using a SPAD-502 meter (Minolta Co., Osaka, Japan).

The pH of the nutrient solution was checked daily with a pHmeter (827 pHlab. Methrohm). In the experiment to follow the evolution of the pH in the nutrient solution, this solution was not changed throughout the experiment. Nutrients were provided refilling the containers with fresh nutrient solution at least once a week depending on the plants needs.

Iron reductase activity measurements

The ferric chelate reductase (FC-R) activity was monitored in both genotypes in vivo in the whole root system following the protocol adapted to the *Prunus* genus by Gogorcena et al. (2000).

120 FC-R activity was measured in plants submitted to Control and Deficient treatments for five and six
121 days, and in plants resupplied 1 and 2 days with 180 μM Fe (III)-EDTA after 4 days of iron
122 shortage.

123 The FC-R activity was also measured in 2 cm excised root tips including the subapical root area
124 from plants of Felinem and P 2175 growing in Control and Deficient treatments during 16 or 17
125 days of iron depletion and in roots of plants submitted to the Inductor treatment growing 15 days
126 without iron and resupplied during 1 and 2 days with 180 μM Fe (III)-EDTA.

127 The visualization and localization of the FC-R activity in roots of Felinem and P 2175 was also
128 carried out in Petri dishes according to the method previously described by Gogorcena et al. (2000).

129 All the analyses were also carried out in the absence of plants or root tips to correct for non-
130 enzymatic Fe reduction.

131 *Proton extrusion*

132 For the proton extrusion detection, root tips of both genotypes, Felinem and P 2175, were
133 incubated in Petri dishes using bromocresol purple as pH indicator as it was described by
134 Gogorcena et al. (2000). Excised root tips from Control and Deficient plants growing without iron
135 for 16 days, and plants from the Inductor treatment resupplied 1 day with 180 μM Fe (III)-EDTA
136 after 15 days of depletion, were placed in the Petri dishes and incubated for 60 minutes.

137 *RNA isolation*

138 Total RNA from excised roots tips of plants growing hydroponically under the treatments above
139 described was isolated following the protocol described by Chang et al. (1993). RNA was treated
140 with RNasin ® Plus RNase Inhibitor (Promega Corporation, USA) to remove residual genomic
141 DNA, and it was cleaned up with the RNeasy Mini kit (QIAGEN Group, USA).

142 Complementary DNA was prepared from 1 μg of total purified RNA with SuperScript™ II
143 Reverse Transcriptase (Invitrogen Corporation, USA) according to the manufacturer's instructions.
144 cDNA was diluted 1:100 for real-time PCR reactions.

145 *Primer design and real-time PCR*

Sequences published in the NCBI (http://www.ncbi.nlm.nih.gov/page) of the *FRO2* and *IRT1* genes from Arabidopsis, cucumber, *Medicago truncatula*, pea and tomato (Eide et al., 1996; Robinson et al., 1999; Eckhardt et al., 2001; Vert et al., 2002; Waters et al., 2002; Li et al., 2004; López-Millán et al., 2004; Waters et al., 2007) and sequences of the *AHA2* gene from Arabidopsis and cucumber (Santi et al., 2005; Santi and Schmidt, 2009) were selected for the *in silico* search of sequences related with the iron metabolism in *Prunus*. Sequence alignment analysis was performed with CAP (http://bioinfo.hku.hk/services/analyseq/cgi-bin/cap_in.pl) and ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) to identify the homologous regions among the selected sequences. These regions were used to perform BLAST searches against the *Prunus* EST database. Sequences with the largest identity were chosen to design degenerate primers for the conserved sites using Primer3 software (Rozen and Skaletsky, 2000). Several primers pairs were designed for each region with large identity. Finally, the following primers were chosen for their differential expression among genotypes: *PFRO2*-fw (5'-CACGTTTCAACACCAAGGCA-3'), *PFRO2*- rev (5'-TCAAACTCACCTCCGCATCT-3'), *PIRT1*-fw (5'-TGCATTGCTCAGGCAGGATT-3'), *PIRT1*-rev (5'-AGCCCTCCAAGATTAGGGCATT-3´), *PAHA2*-fw (5'-GATTGAGAAGGCTGATGGCTTTGCTG-3'), *PAHA2*-rev (5'-TCGTTAACACCATCTCCGGTCATTCC-3') with a temperature of annealing (TA) of 56ºC for *PFRO2* and 60ºC for *PIRT1* and *PAHA2*. The single PCR product length for these three primers pairs were 101, 89 and 120 bp, respectively.

The PCR mixture contained 2 µl of diluted cDNA (corresponding to 1 ng of starting amount of RNA), 10 µl of Quantimix SYBR Green PCR Master Mix (Biotools Laboratories) and 200 nM of each primer in a final volume of 20 µl.

The real-time PCR was performed by the iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories). The results were analyzed using the iCycler iQ Optical System Software (version 3.0a). The efficiency of the amplification was assessed relative to the standard curve method using

series of 10-fold dilutions of cDNA from roots submitted to the Control treatment. Expression levels were calculated relative to the values obtained by geNorm v.3.5 (Vandesompele et al., 2002) using one of the three housekeeping genes (Actin1, Actin 2/7 and Ubiquitin 1) studied. The experiment was carried out from a bulk of samples submitted to the different treatments from at least five different biological replicates and three technical replications. Gene expression data were evaluated by Student's t test with the program SPSS 17.0 (SPSS, Inc, Chicago USA). The normalized values were compared every day with the corresponding Control treatment. For each gene, the expression level for the Control treatment was set to 1.

Results

Iron deprivation affected the Felinem and P 2175 plants inducing physiological changes and differential expression of genes involved in iron homeostasis. However, except for the loss of pigmentation produced along the experiments, the external appearance of the plants was not affected by the lack of this micronutrient (data not shown). The root did not show an increase of lateral roots and/or root hairs, and in the aerial system, the shoot growth was similar between plants submitted to Control and Deficient treatments.

I. Physiological response

In plants of Felinem and P 2175 submitted to iron deprivation, diverse physiological changes were observed. Firstly, in both genotypes the leaf chlorophyll concentration, measured as SPAD readings, decreased gradually throughout the experiment (Fig. 1). SPAD values for Felinem, with red leaves, were always higher than values obtained for P 2175 with green leaves. The SPAD values decreased over the time reaching values associated with severe chlorosis symptoms (SPAD readings below 10) after 10 and 16 days of iron deprivation for P 2175 and Felinem, respectively (Fig. 1). Plants from both genotypes submitted to the Control treatment increased their SPAD readings to values close 40 the 17th day.

Figure 1

Figure 2

197 At the same time, the pH of the nutrient solution for each genotype was monitored. The pH of
198 the Control nutrient solution increased gradually in both Felinem and P 2175 plants, while the
199 values obtained in the iron free nutrient solution showed different pattern between genotypes from
200 the sixth day of iron shortage. The pH of the nutrient solution for Felinem in both treatments,
201 Control and Deficient, was similar over time, increasing gradually to reach values close to 7.0 at the
202 17^{th} day of iron depletion (Fig. 2A). On the contrary, the pH of the iron-free nutrient solution for P
203 2175 remained constant from the sixth to the ninth day, and then, the pH started to decrease
204 dropping down to values near 4.0 at the end of the experiment (Fig. 2B). The sharp decrease in pH
205 values was coincident in time with the apparition of severe chlorosis symptoms observed in leaves
206 after nine days of iron shortage (Fig. 1). After 15 days of iron removal from the nutrient solution,
207 the proton extrusion was also detected in the subapical zone of the roots from P 2175 deficient
208 plants. The proton extrusion was observed in Petri dishes when media turned to yellow due to the
209 pH decrease as indicated by bromocresol purple (Fig. 2B).
210 The FC-R activity was measured in roots of intact plants submitted to different iron supply
211 treatments. Felinem plants growing without iron for five days had lower FC-R activity than Control
212 plants. After four days of iron deprivation and resupply with 180 μM Fe (III)-EDTA, the FC-R
213 activity was induced 4-fold in Felinem after 1 and 2 days of the iron addition (Fig. 3A). In contrast, Figure 3
214 no induction of the FC-R activity was found in roots of intact P 2175 plants in any of these
215 conditions. For that reason, the FC-R activity was later measured in excised roots of plants
216 submitted to longer periods of iron starvation. The use of root tips allowed the analysis of the active
217 parts giving more accurate information. Surprisingly, root of P 2175 plants submitted to iron
218 deprivation for at least 15 days showed induction of the FC-R activity 4-fold higher than Control
219 plants. Moreover, the induction of the ferric chelate reductase activity decreased after one and two
220 days of adding 180 μM Fe (III)-EDTA (Fig. 3B). Also, it was possible to localize the FC-R activity
221 at the subapical zone of the root tip of plants submitted to 15 days of iron absence, by the reddish
222 coloration of the media surrounding this area due to the iron reduction (data not shown).

II. Gene expression

The expression of three genes related with iron metabolism in roots, *PFRO2, PIRT1* and *PAHA2* was analyzed in the two *Prunus* genotypes submitted to different iron treatments. In the experiment time course, differences in the gene expression were observed. First, the expression of these genes was analyzed in roots of plants submitted to iron deficiency for short periods of time (four, five and six days of iron deprivation) and the corresponding resupply with 180 μM Fe (III)-EDTA at one and two days.

The expression of two of the three analyzed genes, *PFRO2* and *PIRT1*, in the Felinem genotype increased under iron deprivation conditions (Fig. 4A and B) although the level of expression was higher in the *PIRT1* gene. After five days of iron depletion, the expression of *PFRO2* in the roots of Felinem plants was 12-fold induced in deficient plants. In this genotype, roots of plants resupplied with iron after four days of deficiency showed a 5-fold induction after 24 hours compared with the expression for control plants with a continuous iron input (Fig. 4A). Furthermore, the expression of the iron transporter was rapidly induced in this genotype after iron starvation. The expression was 100-fold higher in deficient plants after four and five days without this micronutrient in the nutrient solution. The expression of this iron transporter gene was also stimulated in plants resupplied with iron after a short period of lack of iron (Fig. 4B). The expression of the gene encoded for the membrane ATPase, *PAHA2*, was only significantly induced after four days of deficiency in Felinem (Fig. 4C). The expression of these genes in plants submitted to lack of iron for longer periods of time showed not significant differences among treatments.

Figure 4

We did not found any significant differential gene expression in *PFRO2*, *PIRT1* and *PAHA2* for the P 2175 genotype submitted to 4, 5 and 6 days of iron deprivation in any of the treatments. The expression of these genes in roots of plants submitted to longer periods of iron shortage (15, 16 and 17 days of iron deprivation), and later resupplied with 180 μM Fe (III)-EDTA during one and two days was studied. In these new conditions, the expression of *PFRO2* in the P 2175 genotype did not show any significant differences through the experiment in any treatment (Fig. 5A). However, the

Figure 5

249 expression of the gene encoded for the iron transporter (*PIRT1*) was highly induced in roots of
250 Deficient plants after 16 (12-fold) and 17 days (9-fold) of iron suppression (Fig. 5B). The
251 expression of this gene was also induced (12-fold) in the roots of plants submitted to the Inductor
252 treatment at one and two days of iron resupply (Fig. 5B). Moreover, significant differences with a
253 2-3 fold induction in the expression of *PAHA2* in P 2175 roots were observed in plants submitted to
254 iron deprivation after 16 and 17 days of treatment (Fig. 5C).
255
256 **Discussion**
257 *Physiological performance of the rootstocks under iron deficiency conditions*
258 Results showed that both genotypes were iron efficient species able to activate several
259 mechanisms of response to deal with iron shortage. However, in contrast with other species, except
260 for the visual symptoms of Fe deficiency (leaf chlorosis), morphological changes to increase the
261 uptake surface in roots were not found in these two *Prunus* rootstocks under our experimental
262 conditions. The absence of apparent morphological changes was previously reported in *Prunus*
263 plants (Romera et al., 1991; Gogorcena et al., 2000) and other woody plants such as *Quercus suber*
264 (Gogorcena et al., 2001).
265 It is noteworthy the different physiological responses activated in both genotypes under iron
266 deficient conditions. The FC-R activity in Felinem increased in roots of plants submitted to the
267 Inductor treatment, after only 4 days of iron depletion and resupply with iron for one and two days,
268 in agreement with results obtained in similar experimental conditions by Jiménez et al. (2008).
269 According with these authors, Felinem could be classified as tolerant to iron chlorosis The
270 performance of Felinem, under field growing conditions, confirmed its tolerance, probably due to
271 the influence of almond pedigree in its genotype (Felipe, 2009).
272 On the other hand, the performance of P 2175 under controlled iron deficient conditions was still
273 unknown, even though plum rootstocks have been previously described in general as tolerant to this
274 nutritional disorder in the field (Socias i Company et al., 1995). The response found in the P 2175

genotype under iron deficiency was atypical either for the FC-R activity or the proton release never detected before in our hydroponic conditions. However, the rhizosphere acidification, as a part of the response mechanism for the Strategy I plants against lack of iron, has been reported in other plum genotypes under different growing conditions (Romera et al., 1991), and in other woody plants as pear (Donnini et al., 2009), peach-almond hybrids (Molassiotis et al., 2006), cork oak (Gogorcena et al., 2001), grapevine (Jiménez et al., 2007) as well as in herbaceous species (López-Millán et al., 2001; Santi et al., 2005).

It is possible that the mechanisms resulting from the total iron deficiency in controlled conditions may differ from that resulting from the natural field conditions. But, due to the difficulty of reproduce exactly the calcareous soil conditions with roots of woody species, the method based on the induction of ferric chelate reduction capacity in deficient peach rootstocks was established as one of the best to evaluate chlorosis tolerance in *Prunus* species grown under hydroponic conditions (Gogorcena et al., 2004; Molassiotis et al., 2006; Jiménez et al., 2008). It has been previously reported that the measurement of the enzyme FC-R activity provides a better screening ability than H^+ ion release (Gogorcena et al., 2004; Jiménez et al., 2008). However, the results obtained in this work have suggested the importance of checking both responses due to the activation of different mechanisms under iron shortage conditions depending on the genotype. Thus, Felinem would use the iron reduction as the main mechanism to capture the iron from the soil while in P 2175 the mechanism of response would start with the acidification of the medium synchronized with the loss of chlorophyll in leaves as a signal of iron deficiency. The association between the induction of the FC-R activity and the increase of H^+ extrusion, as a mechanism of regulation, was reported in the peach-almond GF 677 rootstock submitted to iron deprivation (Molassiotis et al., 2006). Probably, in the plum P 2175, the global iron status of the plant might be involve at the beginning of the response with a shoot to root signal activated by the loss of iron in the aerial part of the plant as previously described in other species such as Arabidopsis (Vert et al., 2003) and peas (Grusak and Perzeshgi, 1996). The iron status of the plant could be also associated to the root apoplasmic iron

301 that can be mobilized and translocated to the shoots (Longnecker and Welch, 1990). Thus, chlorosis
302 symptoms occurrence could be related with the capacity of the genotypes to store iron at the root
303 apoplasm and its re-mobilization to the upper plant parts. The Myrobalan P 2175 would be more
304 efficient in the iron re-mobilization in response to a signal of the leaves when the chlorosis become
305 severe. The importance for the iron uptake regulation of the shoot-to root communication in higher
306 plants was already described by several authors (Grusak and Pezeshgi, 1996; Schmidt et al., 1996).
307 *Differential gene expression of PFRO2, PIRT1 and PAHA2 under iron deficiency conditions*
308 The identification of the genes involved in the physiological responses would imply the better
309 understanding of the iron chlorosis tolerance regulation. Our results showed different expression in
310 the three studied genes in both *Prunus* genotypes. The induction of the *PFRO2* expression, after 5
311 days in roots of Felinem, in response to lack of iron has been already reported in other plant species
312 growing in a range between 3 to 12 days without iron in the nutrient solution (Robinson et al., 1999;
313 Waters et al., 2002; Connolly et al., 2003; Vert et al., 2003; Ding et al., 2009). The decrease
314 observed in the *PFRO2* gene expression after transferring the plants to iron sufficient conditions
315 was also described in peanut one day resupplied plants after 7 days without iron (Ding et al., 2009).
316 The *PFRO2* gene expression in P 2175 did not show any significant differences along the
317 experiment probably due to the time period not long enough to detect changes in its expression.
318 Once the iron is reduced, the transport into the plant is the next step, and *IRT1* is the gene
319 responsible for iron uptake in roots (Vert et al., 2001; 2002). The expression of the *PIRT1* gene in
320 Felinem was strong and early on the time course of the experiment suggesting a short-term response
321 to lack of iron. The mechanism of response was rapidly activated in absence of iron. The rapid
322 stimulation of *IRT1* gene expression in response to Fe shortage was already reported in Arabidopsis
323 plants by Buckhout et al. (2009). Furthermore, the expression of *PIRT1* in Felinem roots decreased
324 when the plants were transferred to iron sufficient nutrient solution, probably to avoid the
325 accumulation of this metal in toxic levels (Connolly et al., 2002) and imbalances in ion distribution
326 (Buckhout et al., 2009).

The induction of the *PIRT1* gene expression in the roots of P 2175 submitted to iron deprivation at least for 16 days was coincident in time with the detection of the main physiological mechanisms of response. The level of expression was considerably lower when compared with the Felinem genotype. Furthermore, the *PIRT1* gene expression remained constant under resupply conditions. This slower response could be explained by a better adaptation of P 2175 to Fe-deficient conditions. The differences in the level of expression could be due to dual control of the transporter gene regulated by both systemic and local signals (Vert et al., 2003), suggesting in the case of P 2175 a long-term response based on the whole plant iron status. On the other hand, the rapid response to Fe shortage in Felinem plants could be due to a short term response to local lack of iron. However, it is unclear how the Fe status of the plant is perceived and which cellular components would be involved in signal perception (Buckhout et al., 2009). In this work, it has been studied only one transporter gene. Nevertheless, other genes could be taking part of the process as the homologous *IRT2* probably involved in the toxicity prevention by compartmentalization of iron (Vert et al., 2001) and in the remobilization of this nutrient into the plant (Vert et al., 2009).

Coordinate regulation of *PFRO2* and *PIRT1* genes expression was observed in Felinem roots when they were submitted to few days without iron. This co-regulation between these two genes was already reported (Connolly et al., 2003; Vert et al., 2003). Early expression of these genes was also observed in roots of Arabidopsis plants submitted to 7 days of iron deficiency. In those conditions, the most rapidly responding transcript encoded the iron uptake transporter and the number of transcripts detected was disproportionally high (Buckhout et al., 2009). The results reported by these authors agreed with the results obtained in both *Prunus* genotypes despite the different time of the response activation. The highest expression level of *PIRT1* compared with *PFRO2* in both genotypes supported the idea of iron reductase as the rate limiting step in iron uptake (Grusak et al., 1990). Nevertheless, *PIRT1* could be also involved in other divalent cation transport, when the availability of Fe in the media is low (Buckhout et al., 2009). Furthermore, the expression of *PIRT1* was rapidly reduced after addition of iron to the media and the recovery of

sufficient iron levels, probably to avoid accumulation of iron at toxic levels (Connolly et al., 2002) as found in Felinem.

Finally, the expression analysis of the gene *PAHA2* was not significantly induced in our experimental conditions. The plasma membrane ATPase plays a major role in plant physiology process providing the energy to activate the ion and metabolite transport (Palmgren, 2001). Some of the *AHA* genes encoded for ATPases have been described as Fe-regulated (Santi et al., 2005; Waters et al., 2007; Santi and Schmidt, 2009). Until now, acidification as a response to iron deficiency was not detected in our hydroponic working conditions (Gogorcena et al., 2004; Jiménez e al., 2008) probably due to the short time of the experiment length to find this response when plum based rootstocks were used. So, when plants of the plum rootstock P 2175 were maintained 15 days under iron deficiency, enough time to develop severe chlorosis symptoms, the response mechanisms were activated. Thus, the expression of *PAHA2* in P 2175 after 16 days of treatment slightly increased in Fe-Deficient plants and it was inhibited by resupply. The highest expression of *PAHA2* gene in these conditions coincided with the lower pH values, probably due to the proton extrusion as a mechanism to improve the iron acquisition as it was reported in cucumber (Santi et al., 2005; Santi and Schmidt, 2009). On the other hand, just like Felinem, in other Strategy I species the acidification of the rhizosphere do not occur or occur only in a lower degree as in soybean, supporting the idea that every plant species can modulate the activation of biochemical mechanisms related to specific environmental conditions or according to their intrinsic characteristics (Zocchi et al., 2007).

However, the *PAHA2* expression levels in P 2175 Fe-Deficient roots were lower than the expected ones, which could be explained by the existence of different isoforms of this gene differently regulated under iron shortage conditions, and it could be highly related with the proton extrusion to the media as described in Arabidopsis (Santi and Schmidt, 2009).

In summary, this paper include the physiological performance of two *Prunus* genotypes, used as progenitors in breeding programs, grown under iron deficient conditions. The two genotypes showed tolerance to iron chlorosis but the mechanisms used to overcome the lack of this essential element were different. The genes involved in iron metabolism were differentially expressed, and at different time course in both genotypes, and were in agreement with the biochemical response observed. The almond-peach hybrid, Felinem, activated the expression of the ferric chelate reductase and the iron transporter genes. A local signal of lack of iron seems to induce a quick response of the transporter and later on the FC-R gene. The response to iron absence of the Myrobolan plum, P 2175, was delayed compared with Felinem suggesting than the gene expression could be induced by a longer distant signal based on the iron status and remobilization of the entire plant.

The identification of genes differentially expressed in *Prunus* rootstocks under iron deprivation would represent an excellent advantage in the knowledge of tolerance to iron chlorosis in fruit trees. The use of these genes to develop molecular markers associated with the iron chlorosis tolerance could be applied as a selection tool in breeding programs. However, further studies should be done to completely understand the mechanism of response activated under iron limited conditions in rootstocks from the *Prunus* genus.

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References

404 Buckhout TJ, Yang TJM, Schmidt W. Early iron-deficiency-induced transcriptional changes in
405 Arabidopsis roots as revealed by microarray analyses. BMC Genomics 2009;10:147.
406 Chang S, Puryear J, Cairney J. A Simple and efficient method for isolating RNA from pine trees.
407 Plant Mol Biol Rep 1993;11:113-16.
408 Cinelli F, Viti R. Practical use of root cation exchange capacity as a predictive marker of lime-
409 induced chlorosis tolerance in *Prunus cerasifera* L. rootstocks. J Plant Nutr 1995;18:65-75.
410 Connolly E L, Campbell NH, Grotz N, Prichard CL, Guerinot ML. Overexpression of the *FRO2*
411 ferric chelate reductase confers tolerance to growth on low iron and uncovers posttranscriptional
412 control. Plant Physiol 2003;133:1102-10.
413 Connolly E L, Fett J P, Guerinot ML. Expression of the *IRT1* metal transporter is controlled by
414 metals at the levels of transcript and protein accumulation. Plant Cell 2002;14:1347-57.
415 Ding H, Duan L, Wu H, Yang R, Ling H, Li W-X, Zhang F. Regulation of *AhFRO1*, an Fe (III)-
416 chelate reductase of peanut, during iron deficiency stress and intercropping with maize. Physiol
417 Plant 2009;136:274-83.
418 Dirlewanger E, Cosson P, Howad W, Capdeville G, Bosselut N, Claverie M, Voisin R, Poizat C,
419 Lafargue B, Baron O, Laigret F, Kleinhentz M, Arús P, Esmenjaud D. Microsatellite genetic
420 linkage maps of myrobalan plum and an almond-peach hybrid-location of root-knot nematode
421 resistance genes. Theor Appl Genet 2004;109:827-38.
422 Donnini S, Castagna A, Ranieri A, Zocchi G. Differential responses in pear and quince genotypes
423 induced by Fe deficiency and bicarbonate. J Plant Physiol 2009;166:1181-93.
424 Eckhardt U, Marques AM, Buckhout TJ. Two iron-regulated cation transporters from tomato
425 complement metal uptake-deficient yeast mutants. Plant Mol Biol 2001;45:437-48.
426 Eide D, Broderius M, Fett J, Guerinot ML. A novel iron-regulated metal transporter from plants
427 identified by functional expression in yeast. Proc Natl Acad Sci USA 1996; 93:5624-28.
428 Felipe AJ. 'Felinem', 'Garnem', and 'Monegro' almond x peach hybrid rootstocks. HortScience
429 2009;44:196-97.

Gogorcena Y, Abadía J, Abadía A. Induction of in vivo root ferric chelate reductase activity in fruit tree rootstock. J Plant Nutr 2000;23:9-21.

Gogorcena Y, Molias N, Larbi A, Abadía J, Abadía A. Characterization of the responses of cork oak (*Quercus suber*) to iron deficiency. Tree Physiol 2001;21:1335-40.

Gogorcena Y, Abadía J, Abadía A. A new technique for screening iron-efficient genotypes in peach rootstocks: Elicitation of root ferric chelate reductase by manipulation of external iron concentrations. J Plant Nutr 2004;27:1701-15.

Grusak MA, Pezeshgi S. Shoot-to-root signal transmission regulates root Fe (III) reductase activity in the dgl mutant of pea. Plant Physiol 1996;110:329–34.

Grusak MA, Welch RM, Kochian LV. Does iron deficiency in *Pisum sativum* enhance the activity of the root plasmalemma iron transport protein? Plant Physiol 1990;94:1353-57.

Jiménez S, Gogorcena Y, Hévin C, Rombola AD, Ollat N. Nitrogen nutrition influences some biochemical responses to iron deficiency in tolerant and sensitive genotypes of Vitis. Plant Soil 2007; 290:343-55.

Jiménez S, Pinochet J, Abadía A, Moreno M, Gogorcena Y. Tolerance response to iron chlorosis of *Prunus* selections as rootstocks. HortScience 2008;43:304-9.

Li L, Cheng X, Ling H-Q. Isolation and characterization of Fe (III)-chelate reductase gene *LeFRO1* in tomato. Plant Mol Biol 2004;54:125-36.

Longnecker N, Welch RM. Accumulation of apoplastic iron in plant roots. Plant Physiol 1990;92:17-22.

López-Millán A-F, Morales F, Gogorcena Y, Abadía A, Abadía J. Iron resupply-mediated deactivation of Fe-deficiency stress responses in roots of sugar beet. Aust J Plant Physiol 2001;28:171-80.

López-Millán A-F, Ellis DR, Grusak MA. Identification and characterization of several new members of the ZIP family of metal ion transporters in *Medicago truncatula* Plant Mol Biol 2004;54:583-96.

456 Marsh HV, Evans HJ, Matrone G. Investigations of the role of iron in chlorophyll metabolism. II
457 Effect of iron deficiency on chlorophyll synthesis. Plant Physiol 1963;38:638-42.
458 Molassiotis A, Tanou G, Diamantidis G, Patakas A, Therios I. Effects of 4-month Fe deficiency
459 exposure on Fe reduction mechanism, photosynthesis gas exchange, chlorophyll fluorescence
460 and antioxidant defense in two peach rootstocks differing in Fe deficiency tolerance. J Plant
461 Physiol 2006;163:176-85.
462 Mukherjee I, Campbell NH, Ash JS, Connolly EL. Expression profiling of the Arabidopsis ferric
463 chelate reductase (*FRO*) gene family reveals differential regulation by iron and copper. Planta
464 2006; 223:1178-90.
465 Palmgren MG. Plant plasma membrane H+-ATPases: Powerhouses for nutrient uptake. Annu Rev
466 Plant Physiol Plant Mol Biol 2001;52:817-45.
467 Robinson NJ, Procter CM, Connolly EL, Guerinot ML. A ferric-chelate reductase for iron uptake
468 from soils. Nature 1999;397:694-97.
469 Romera FJ, Alcántara E, De la Guardia MD. Characterization of the tolerance to iron chlorosis in
470 different peach rootstocks grown in nutrient solution: II Iron-stress response mechanisms. Plant
471 Soil 1991;130:121-25.
472 Römheld V. Different strategies for iron acquisition in higher plants. Physiol Plant 1987;70:231-34.
473 Rozen S, Skaletsky HJ. Primer3 on the WWW for general users for biologist programmers. In:
474 Krawetz S, Misener S, editors. Bioinformatics Methods and Protocols: Methods in Molecular
475 Biology. Humana Press;2000,p 365-86.
476 Santi S, Cesco S, Varanini Z, Pinton R. Two plasma membrane H^+-ATPase genes are differentially
477 expressed in iron-deficient cucumber plants. Plant Physiol Biochem 2005;43:287-92.
478 Santi S, Schmidt W. Dissecting iron deficiency-induced proton extrusion in Arabidopsis roots. New
479 Phytol 2009;183:1072-84.
480 Schmidt W, Boomgaarden B, Ahrens V. Reduction of root iron in *Plantago lanceolata* during
481 recovery from Fe deficiency. Physiol Plant 1996;98:587-93.

Schmidt W. Mechanisms and regulation of reduction-based iron uptake in plants. New Phytol 1999;141:1-26.

Socias i Company R, Gómez Aparisi J, Felipe AJ. A genetical approach to iron chlorosis in deciduous fruit trees. In: Abadia J, editor. Iron nutrition in soils and plants. Dordrecht: Kluwer;1995, p 167-74.

Tagliavini M, Rombola AD. Iron deficiency and chlorosis in orchard and vineyard ecosystems. Eur J Agr 2001;15: 71-92.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3:research0034.0031-0034.0011.

Vert G, Briat J-F, Curie C. Arabidopsis *IRT2* gene encodes a root-periphery iron transporter. Plant J 2001;26:181-89.

Vert G, Grotz N, Dédaldéchamp F, Gaymard F, Guerinot ML, Briat J-F, Curie C. *IRT1*, an Arabidopsis transporter essential for iron uptake from the soil and for plant growth. Plant Cell 2002;14:1223-33.

Vert GA, Briat J-F, Curie C. Dual Regulation of the Arabidopsis high-affinity root iron uptake system by local and long-distance signals. Plant Physiol 2003;132:796-804.

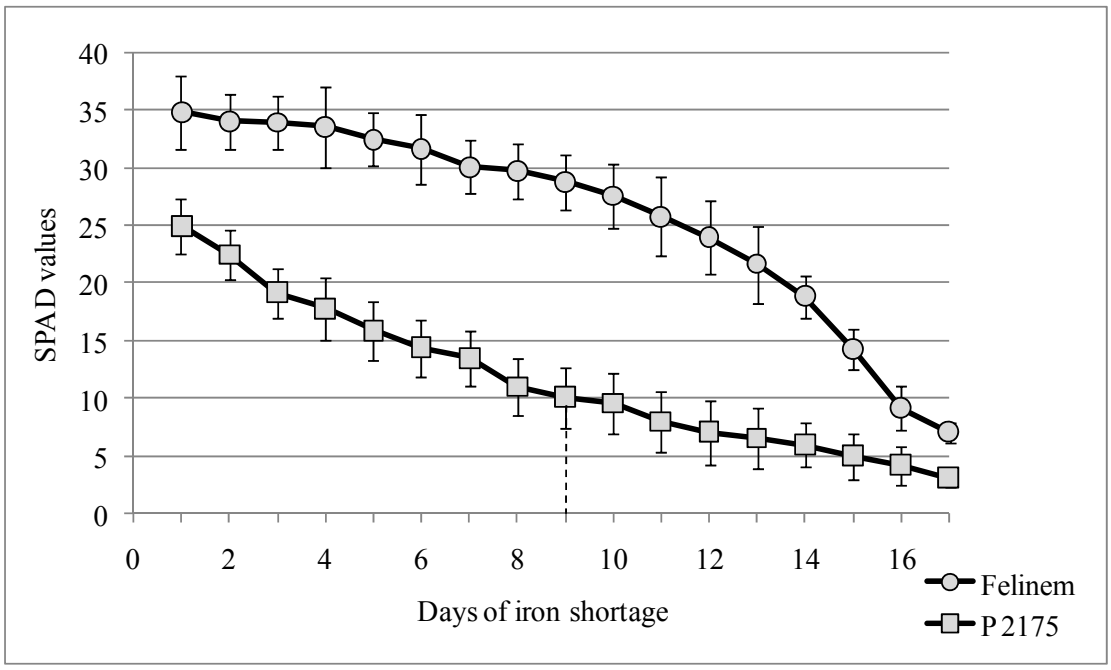
Vert G, Barberon M, Zelazny E, Séguéla M, Briat J-F, Curie C. Arabidopsis IRT2 cooperates with the high-affnity iron uptake system to maintain iron homeostasis in root epidermal cells. Planta 2009;229:1171-79.

Waters BM, Blevins DG, Eide DJ. Characterization of *FRO1*, a pea ferric-chelate reductase involved in root iron acquisition. Plant Physiol 2002;129:85-94.

Waters BM, Lucena C, Romera FJ, Jester GG, Wynn AN, Rojas CL, Alcántara E, Pérez-Vicente R. Ethylene involvement in the regulation of the H^+-ATPase *CsHA1* gene and of the new isolated ferric reductase *CsFRO1* and iron transporter *CsIRT1* genes in cucumber plants. Plant Physiol Biochem 2007;45 293-301.

508 Zocchi G, De Nisi P, Dell'Orto M, Espen L, Gallina PM. Iron deficiency differently affects
509 metabolic responses in soybean roots. J Exp Bot 2007;58:993-1000.

510 **Fig. 1**: Time course of leaf SPAD values of the two *Prunus* rootstocks, Felinem and P 2175,
511 submitted to 17 days of iron shortage. Dashed line point out the day where the chlorosis symptoms
512 started to be severe. Data were evaluated with the software SPSS 17.0 (SPSS, Inc, Chicago USA).



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515 **Fig. 2**: Time course of nutrient solution pH plants grown under control (●) and deficient (○)
516 conditions in (A) Felinem and (B) P 2175 genotypes. Acidification of the medium was observed in
517 the yellow area of the subapical zone in deficient roots of P 2175. Dashed line point out the day
518 where the chlorosis symptoms started to be severe.

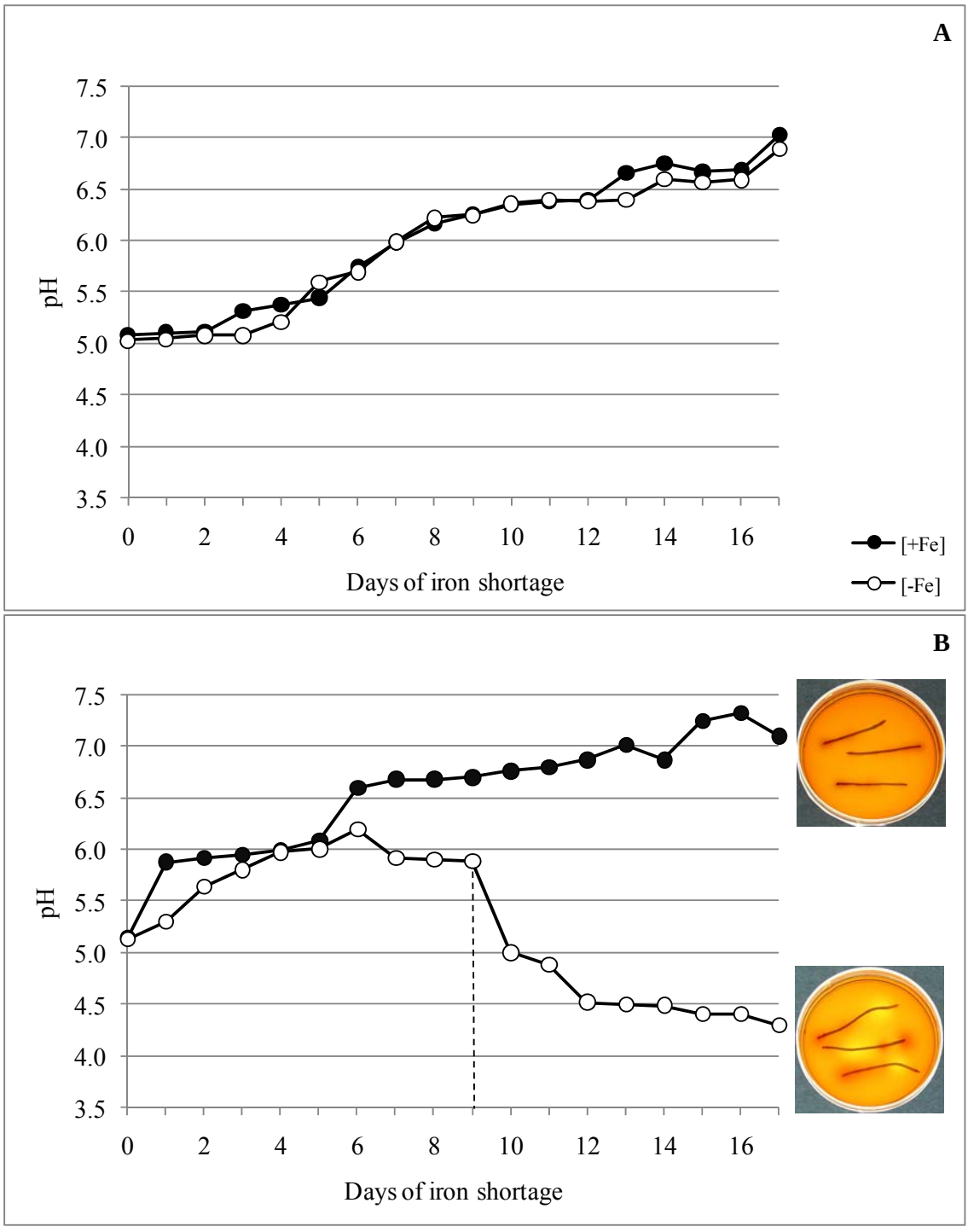

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Fig. 3: Ferric chelate reductase activity (nmol Fe^{2+} min^{-1} g^{-1} FW) of (A) intact roots of Felinem submitted to three treatments: [+Fe] (plants growing under control conditions in 90 μM Fe(III)-EDTA); Deficient [-Fe] (plants growing 5 days in absence of iron); and Inductor (plants growing in iron absence for 4 days and resupplied with 180 μM Fe (III)-EDTA during 1 and 2 days), [180 1d] and [180 2d], and (B) excised roots of P 2175 plants submitted to the treatments: [+Fe] (plants growing under control conditions in 90 μM Fe(III)-EDTA); Deficient [-Fe] (plants growing 16 days in absence of iron); and Inductor (plants growing in iron absence for 15 days and resupplied with 180 μM Fe (III)-EDTA during 1 and 2 days), [180 1d] and [180 2d]. Values are means of three independent experiments. Statistical Student´s t test revealed significant differences at $p \leq 0.05$ (*) and $p \leq 0.01$ (**) compared with control. The statistical analysis were performed using SPSS 17.0 (SPSS, Inc, Chicago USA).

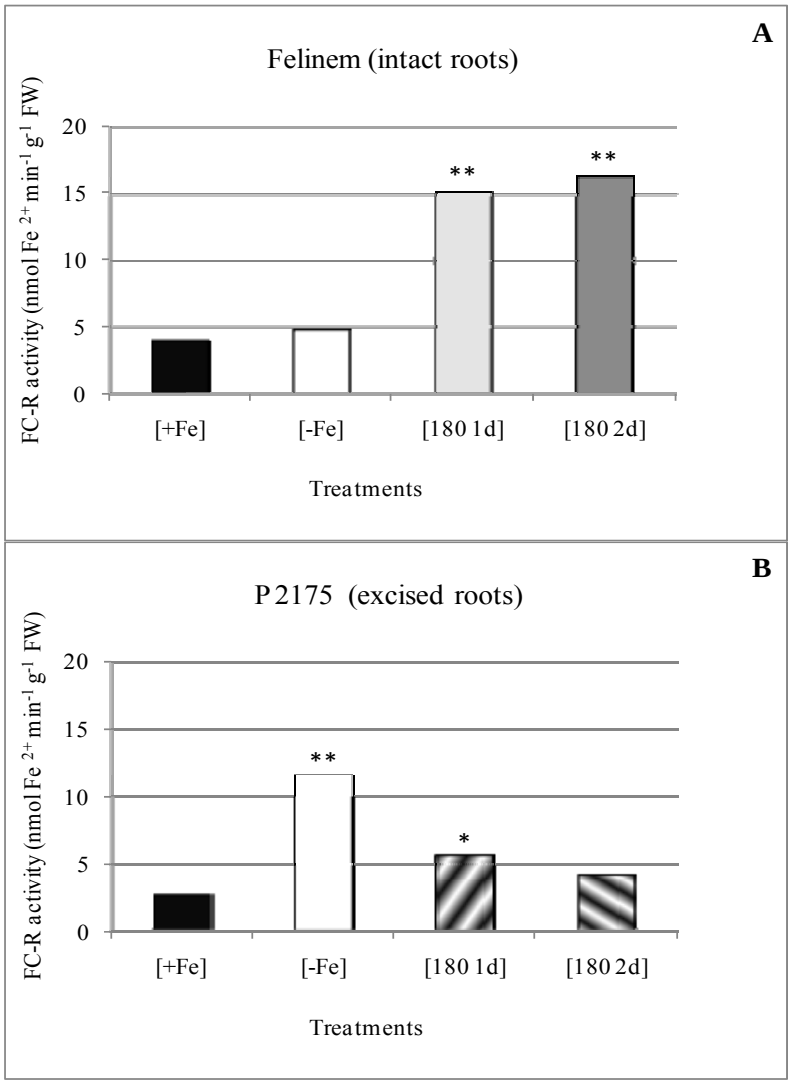

Fig. 4: Time course of the relative expression of (A) *PFRO2*, (B) *PIRT1* and (C) *PAHA2* genes in roots of the Felinem rootstock. The expression analysis was performed in cDNA from roots submitted to three treatments: [+Fe] (plants growing under control conditions in 90 μM Fe(III)- EDTA); Deficient [-Fe] (plants growing 5 days in absence of iron); and Inductor, [180 1d] and [180 2d] (plants growing in iron absence for 4 days and resupplied with 180 μM Fe (III)-EDTA during 1 and 2 days). Values are mean of three replications. Statistical Student´s t test revealed significant differences $p \leq 0.01$ (**) compared with control. The statistical analysis were performed using SPSS 17.0 (SPSS, Inc, Chicago USA).

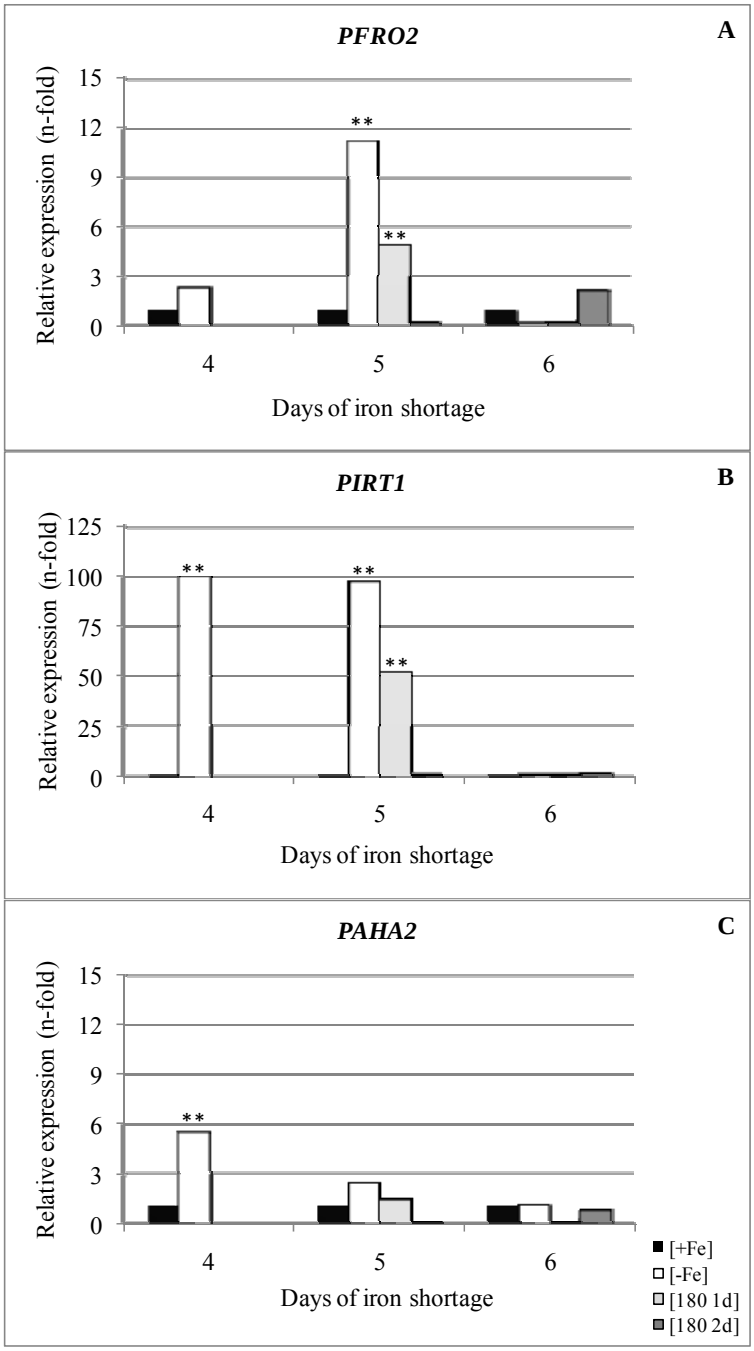

Fig. 5: Time course of the relative expression of (A) *PFRO2*, (B) *PIRT1* and (C) *PAHA2* genes in roots of the P 2175 rootstock. The expression analysis was performed in cDNA from roots submitted to three treatments: [+Fe] (plants growing under control conditions in 90 μM Fe (III)-EDTA); Deficient [-Fe] (plants growing 15, 16 and 17 days in absence of iron); and Inductor, [180 1d] and [180 2d] (plants growing in iron absence for 15 days and resupplied 180 μM Fe (III)-EDTA during 1 and 2 days). Values are mean of three replications. Statistical Student´s t test revealed significant differences at $p \leq 0.05$ (*) and $p \leq 0.01$ (**) compared with control. The statistical analysis were performed using SPSS 17.0 (SPSS, Inc, Chicago USA).